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Detection of Waterborne Pathogens by PCR-reverse Blot Hybridization

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The present study was set to develop comprehensive system for assessing the safety of drinking water using PCR-reverse blot hybridization assay (REBA). The REBA developed in this study can detect waterborne pathogens such as *Shigella* spp., *Salmonella* spp., *Citrobacter* spp., *Enterobacter* spp., *Klebsiella* spp., *Yersinia* spp., *Mycobacterium* spp., *Listeria* spp. at the genus level, and *Escherichia coli*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, *Y. pseudotuberculosis*, *Mycobacterium avium* complex, *M. marinum*, *Enterococcus faecalis*, and *Staphylococcus aureus* at the species level, and *E. coli* O157:H7 at the strain level.

Key Words: Waterborne pathogen, Reverse blot hybridization assay, Nested PCR, Water contamination

INTRODUCTION

Currently, monitoring microbial parameters in water employs microbiological analysis which includes process of sampling and filtration of water followed by cultivation of the chosen microorganism using enrichment and selective media. The whole process usually takes 24 to 72 hours and still may not be able to pick up a number of microorganisms, if appropriate selective media and growth conditions are not used. For that reason, in routine monitoring process, not all of possible water microbial contaminants, but coliform bacteria including *Escherichia coli* that may represent a degree of water contamination have been routinely used for monitoring. However, there are many other species of bacteria that should not be present in the water as water contaminants. Therefore, there have been extensive efforts to develop a method more comprehensive for monitoring all the microbial water contaminants (Bej et al., 1990; Bej et al., 1991; Call et al., 2003).

Recently, molecular methods have been applied for the rapid detection of pathogens in food, soil, and water. These methods have shown high degrees of sensitivity and specificity and do not require the need for complicated cultivation and additional identification process (Bej et al., 1990; Bej et al., 1991; Wilson et al., 2001; Bej AK, 2003; Call et al., 2003). Some of these methods allow detection of specific culturable and/or non-culturable bacteria within serial hours, instead of the days required with the traditional microbiological methods (Tsai et al., 1992; Josephson et al., 1993; Cooper et al., 1997; Mittelman et al., 1997; Jackson et al., 2001; Reynolds et al., 2001; Baudart et al., 2002; Bayardelle et al., 2002; Field et al., 2003).

Most of molecular methods which have been developed so far have been mainly based on the molecular analysis of bacteria with respect to their 16S rRNA or its genes (Clarridge, 2004). These approaches take advantage of usefulness of 16S rRNA, which is a stable taxonomic marker for microorganisms, since genetic variations in 16S rRNA are intergenus and interspecies. Thus, the variation in 16S rRNA can be utilized for the designing of species- and genus-specific genetic markers (Amann et al., 1995; Te Giffel et al., 1997).

Recently, PCR primers and 16S rRNA-based oligonucleotide probes specific for some bacterial pathogens such as *E. coli* (Tsen et al., 1998), *Listeria monocytogenes*,

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Salmonella spp. (Gendel, 1996; Lin and Tsen, 1996; Tsen and Lin 1999), *Staphylococcus* spp. (Couzinet et al., 2005), and *Bacillus* species (Ash et al., 1991) have been reported to be successful in the PCR amplification and the DNA microarray. DNA microarray is especially powerful since it can simultaneously detect multiple targets by one assay (Warsen et al., 2004; Loy et al., 2005). Therefore, DNA hybridization techniques have been reported to be successful for the detection and identification of waterborne pathogens (Kim et al., 2003; Chiang et al., 2006). However, even though DNA microarray is an attractive method, it is still too expensive for many practical settings.

On the other hand, reverse blot hybridization assay (REBA) employing multiple target probes is affordable in many practical settings, since it is relatively simple, cheap, and yet still as informative as DNA microarray. That is, REBA is readily applicable to small clinical laboratories. Several studies have reported the development of REBA, but those studies targeted no more than 6 bacterial species (Kim et al., 2003; Chiang et al., 2006) contrary to the need for more targets.

Therefore, the present study was set up to develop PCR-REBA which can be more comprehensive for detection of waterborne pathogens. Here we report development of new PCR-REBA targeting 1 strain (*E. coli* O157:H7), 10 species (*E. coli*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, *Y. pseudotuberculosis*, *Mycobacterium avium* complex and *M. marinum*, *Enterococcus faecalis*, *Staphylococcus aureus*) and 8 genus (*Shigella* spp., *Salmonella* spp., *Citrobacter* spp., *Enterobacter* spp., *Klebsiella* spp., *Yersinia* spp., *Mycobacterium* spp., *Listeria* spp.) of waterborne pathogens simultaneously. New PCR-REBA assays developed by this approach and evaluation of the new method for the potential usefulness in diagnosing waterborne pathogens in environmental samples are described.

MATERIALS AND METHODS

Bacterial strains and cultivation

The bacterial strains used in this study were *E. coli* O157:H7 (ATCC 35150, ATCC 43894, DML 411), *Shigella dysenteriae*, *S. sonnei*, *S. flexneri*, *Citrobacter freundii*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, *Yersinia pseudotuberculosis*, *Y. enterocolitica*, *Mycobacterium avium* complex, *M. marinum*, *Enterococcus faecalis*, *L. monocytogenes*, *Staphylococcus aureus*. The bacteria were obtained from clinical microbiology laboratory of Department of Biomedical Laboratory Science, Yonsei University. The bacterial strains were grown overnight at 37℃ in brain heart infusion broth and agar plate (Difco Laboratories, MI, USA). The bacterial genomic DNA was prepared using cethyltrimethyl ammonium bromide method as previously described (Lemarchand et al., 1996). The quality and the quantity of the extracted DNA were determined by electrophoresis using a 1% agarose gel and spectrophotometer.

Sample preparation

A total of seven water samples from diverse sources including tap water, spring water, mineral water, lake, river, effluent and sewage from Gangwon Province in Korea were collected, and handled according to guideline suggested by the Korea Ministry of Environment (Korea Ministry of Environment. 2002).

Within less than 4 hrs after sampling, microorganisms in water samples were harvested by filtration of 200 ml of water on a sandwich filter consisting of a 0.45 μm pore size nitrocellulose membrane (Millipore Corp., Bedford, MA, USA). After filtration, the membrane containing the bacteria was placed on a selective and differential medium (MacConkey agar, Mannitol salt agar, Bile esculin azide agar, Middlebrook 7H11 agar) and incubated at 37℃ for 18 hrs. Standard methods were used for the enrichment, isolation, identification, and biochemical confirmation of waterborne pathogens (Lemarchand et al., 1996). Organisms were identified using appropriate API® strips (BioMerieux SA, Lyon, France).

The bacterial genomic DNA was prepared using cethyltrimethyl ammonium bromide method as previously described (Lemarchand et al., 1996).

Table 1. List of oligonucleotide probes for identification of waterborne pathogens

Probe name	Oligonucleotide sequence (5' to 3')	Modification
Universal probe for 16S rRNA gene	CCAGACTCCTACGGGAGGCAGCAGT	5'-amine
Gram negative bacteria	GCTGGTCTGAGAGGATGAYCA	5'-amine
Gram positive bacteria	AAA ACGGGTGAGTAACACGTGG	5'-amine
E. coli O157:H7	GTAACAGGAAGAAGCTTGCTTCTT	5'-amine
Shigella / *E. coli* spp.	GGGAGTAAAGTTAATACCTTTGCTC	5'-amine
Citrobacter freundii	TAGCACAGAGGAGCTTGCTCCTTGGG	5'-amine
Salmonella / *Citrobacter* spp.	TGTTGTGGTTAATAACCGCAGCAA	5'-amine
Klebsiella pneumoniae	GTTAATAACCTCATCGATTGACGT	5'-amine
Enterobacter / *Klebsiella* spp.	AACGTTAAGGTTAATAACCTTGG	5'-amine
Pseudomonas aeruginosa	GCAGTAAGTTAATACCTTGCTGTT	5'-amine
Yersinia enterocolitica	CCAATAACTTAATACGTTGTTGG	5'-amine
Yersinia pseudotuberculosis	GGTTGAGTTTAATACGCTCAATC	5'-amine
Yersinia spp.	CGGGAAGTAGTTTACTACTTTGCCGG	5'-amine
Mycobacterium avium	TCAAGACGCATGTCTTCTGGTG	5'-amine
Mycobacterium marinum	CCACGGGATTCATGTCCTGT	5'-amine
Mycobacterium spp.	CTTTTGCGGTGTGGGATGGGC	5'-amine
Enterococcus faecalis	ACAGTTTATGCCGCATGGCATAAGAGT	5'-amine
Listeria spp.	AAGAGCTTGCTCTTCCAAAGTTAGTGGC	5'-amine
Staphylococcus aureus	CATATGTGTAAGTAACTGTGCACAT	5'-amine

PCR primers and oligonucleotide probes for PCR-REBA assay

To design specific probe molecules, database of 16S rRNA sequences of diverse types of bacteria were compiled from GenBank database of National Center for Biotechnology Information web site (http://www.ncbi.nlm.-nih.gov), and the sequence data for the target bacteria were aligned by using the Clustral method (http://www.-cmbi.kun.nl/bioinf/tools/clustalws.html).

Partial 16S rRNA sequences flanking variable regions, position 40 to 500 relative to the published *E. coli* 16S rRNA sequence, were used. All primers were synthesized by Bioneer (Daejeon, Korea). While the conserved regions were used to design the PCR primers universal for all the bacterial, divergent regions of 16S rRNA genes were used to design the probe molecules to gram-negative, gram-positive and to each species or genus of bacteria targeted. The probes designed in this study are listed in Table 1.

[1] + Y=C:T, M=C:A, R=G:A, D=G:A:T, K=T:G +

PCR amplification for waterborne pathogens[1]

For the 1st PCR amplification, 16S-SF2 (position 42 to 65, 5'-CR†K†GCY†TAAY†ACATGCAAGTCGA-3') and 16S-RSH-A5 (positions 498 to 520, 5'-TGGCACGD†-AGTTR†GCCGK†K†GCTT-3') were used, and the 16S-SF (positions 50 to 68, 5'AAY†ACATGCAAGTCGAR†CK-3') and 16s-R5H-A (positions 498 to 516, 5'-biotin-ACR†D†-AK†TTRGCCGKK†GCTT-3') were used for 2nd PCR amplification. The amplification reactions were performed with a thermal cycler (GeneAmp PCR® System 2700). The 5' end of reverse primers was biotin labeled. PCR amplification was carried out in a 50 μl reaction containing 10 mM Tris-HCl, pH 9.0, 2 mM $MgCl_2$, 50 mM KCl, 200 μM dNTPs, 20 pmole of each primers, 1 units of *Taq* DNA polymerase (Bioneer Co., Daejeon, Korea), and 200 pg of genomic DNA extracted from bacterial pure culture. The PCR reaction was carried out as either single PCR using one set of primers, or one-tube nested PCR using two sets of primers. For single PCR, 35 cycles were done using program consisted of denaturation at 95℃ for 30 s, annealing at 52℃ for 30 s, and extension at 72℃ for 1 min.

For one-tube nested PCR, the first 15 cycles of PCR were done at the condition as follows: the initial denaturation at 95℃ for 30 s, annealing at 62℃ for 30 s, and extension at 72℃ for 1 min. Subsequently, the 35 cycles of the second PCR were done by the program consisted of denaturation at 95℃ for 30 s, annealing at 52℃ for 30 s, and extension at 72℃ for 1 min.

PCR-reverse blot hybridization assay

The preparation of the membrane containing probe molecules and the reverse blot hybridization was performed using a system that has been described previously (Xiang et al., 2002; Zwart et al., 2003). Optimal probe concentrations were determined by binding varying amounts of the probe in such a way that all the probes resulted in equally intense signals relative to the catchall at a concentration ranging from 1 to 40 pmoles.

For PCR-REBA, hybridization was carried out at 50℃ for 90 min in hybridization solution, and the membrane was washed twice with 2X SSPE/ 0.5% SDS at 62℃ for 10 min. Diluted streptavidin-alkaline phosphatase conjugate (1/2000) was then added and incubated at 42℃ within rolling bottle for 60 min. Finally the membrane was washed twice with 2X SSPE/ 0.5% SDS at 42℃ for 10 min, and with 2X SSPE at RT for 5 min. For detection, the membrane was incubated with CDP-star (Amersharm, Uppsala, Sweden) for 4 min and exposed to Hyperfilm (Amersharm, Uppsala, Sweden) for 60 min.

RESULTS

Development of PCR-REBA for detection of waterborne pathogens

For detection of waterborne pathogens, PCR can be used to amplify DNA from environmental samples, and then the PCR-REBA can be used to identify the amplified products.

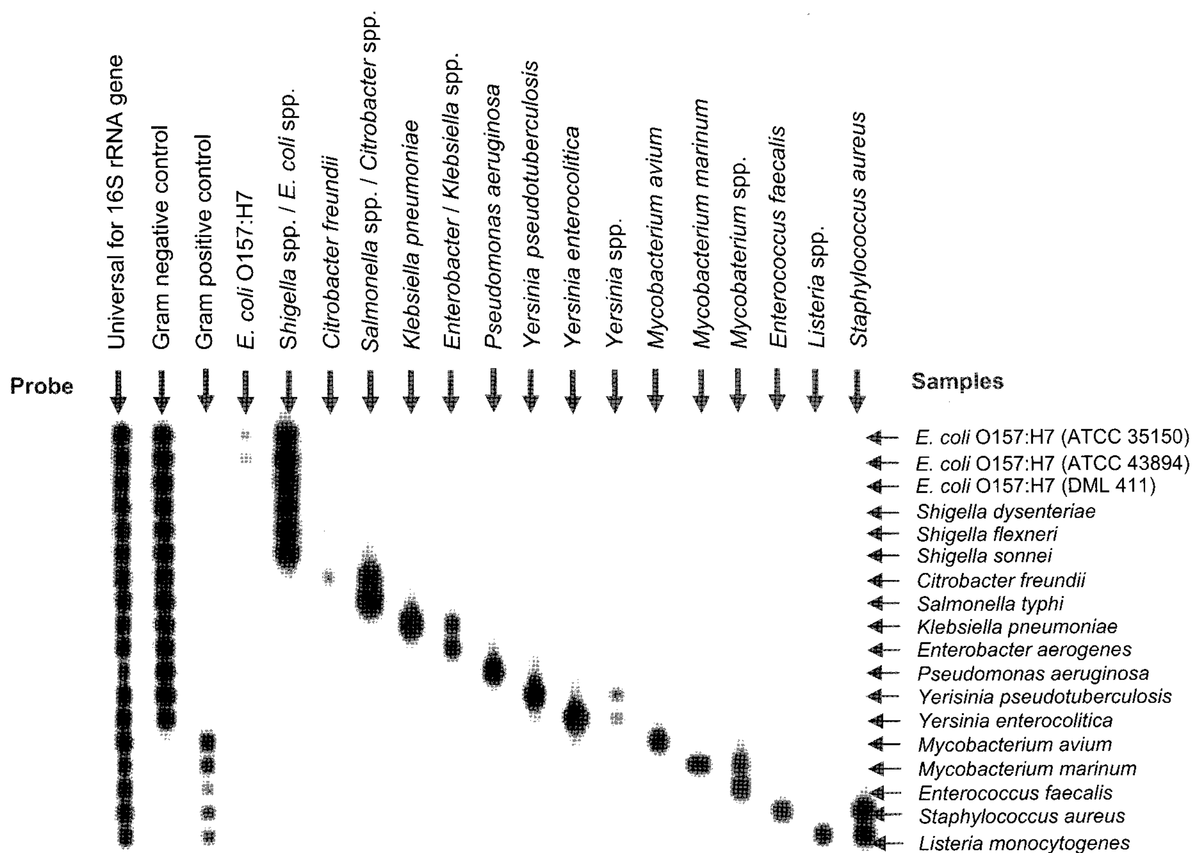

Fig. 1. The specificity of the PCR-REBA. PCR amplicons obtained from amplification of the reference strains of bacterial DNA were hybridized to the membrane. PCR and the hybridization condition are described in the text.

In other words, highly conserved primer sequences (universal 16S rRNA primers) can be used to amplify bacterial DNA followed by hybridization to the membrane composed of pathogen-specific probes. The probes themselves are located within the polymorphic region that is flanked by the conserved primer sequences.

To design specific probe molecules, database of 16S rRNA sequences of diverse types of bacteria were compiled from GenBank database of National Center for Biotechnology Information web site (http://www.ncbi.-nlm.nih.gov), and the sequence data for the target bacteria were aligned by using the Clustral method (http://www.-cmbi.kun.nl/bioinf/tools/clustalws.html).

While the conserved region was used to design the universal PCR primers for all the bacterial, divergent regions of 16S rRNA genes were used to design the probe to gram-negative, gram-positive and to each species or genus of bacteria targeted.

In order to develop PCR-REBA useful for comprehensive monitoring of microbial water contaminants, 18 waterborne pathogens were selected based on their significance as waterborne pathogens. Target bacterial species selected in this study include strains of common waterborne pathogens that may cause diarrhea or vomiting such as *E. coli*, *E. coli* O157:H7, *Shigella* spp., *Salmonella* spp., *C. freundii*, *K. pneumoniae*, *E. aerogenes*, *P. aeruginosa*, *Yersinia* spp., *Y. enterocolitica*, *Y. pseudotuberculosis*, *E. faecalis*, *Listeria* spp., *S. aureus*. *M. avium* complex and *M. marinum* that are major mycobacteria causing diseases by contaminated water and extremely difficult to culture were also targeted. In brief, the probes designed in this study are listed in the Table 1, and results showing the specificity of the probes for detecting each target bacteria are shown in the Fig. 1.

Specificity of the PCR-REBA

Different parameters for PCR-REBA hybridization, such

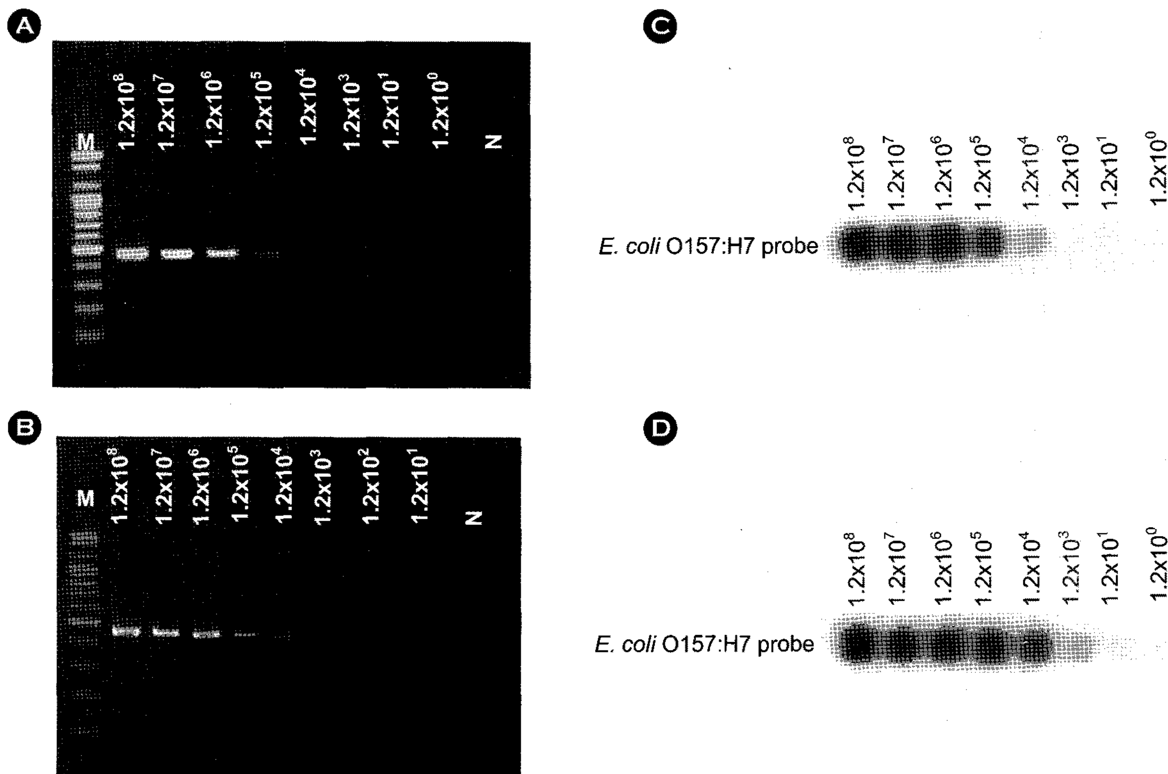

Fig. 2. The sensitivity of the PCR-REBA. (A-B) The sensitivity of the PCR using single and one-tube PCR was compared. (A) single PCR, PCR using one set of primers (16S-SF, 16S-R5H-A) and (B) one-tube nested PCR, PCR using two sets of PCR primers (16S-SF, 16S-R5H-A and 16S-SF2, 16S-R5H-A5). **(C-D)** The sensitivity of the PCR-REBA using PCR amplicons from single (C) and one-tube nested PCR (D) was compared. In order to compare the sensitivity of PCR with that of REBA, PCR amplicons obtained from (A) and (B) were subsequently hybridized to the REBA membrane containing probe for *E. coli* O157:H7. M; 100bp DNA ladder (Bioneer. Co., Daejeon., Korea). (C) shows the PCR-REBA result with (A), and (D) shows the PCR-REBA results with (B).

as temperature, incubation time, salt composition and shaking speed, were optimized for achieving the desired specificity of the PCR-REBA.

As shown in the Fig. 1, hybridization results were in precise agreement with those predicted from the probe sequences. The 9 species-specific probes (*C. freundii*, *K. pneumoniae*, *P. aeruginosa*, *Y. enterocolitica*, *Y. pseudotuberculosis*, *M. avium* complex, *M. marinum*, *E. faecalis*, *S. aureus*), 1 strain-specific probe (*E. coli* O157:H7), and 3 genus-specific probes (*Yersinia* spp., *Listeria* spp., and *Mycobacterium* spp.) hybridized to their targets and did not show any cross reactivity.

On the other hand, some probes were not specific to the relevant genus. For example, *Shigella* spp. and *E. coli*, *Salmonella* spp. and *Citrobacter* spp., *Klebsiella* spp. and *Enterobacter* spp. hybridized to the same probe, since their 16S rRNA sequences are the same. Therefore, all probes demonstrated no cross-reaction with other DNA.

Sensitivity of PCR-REBA

The sensitivity of PCR-REBA using single PCR using one set of primers (16S-SF, 16S-R5H-A) and of one-tube nested PCR using two sets of PCR primers (16S-SF, 16S-R5H-A and 16S-SF2, 16S-R5H-A5) were compared

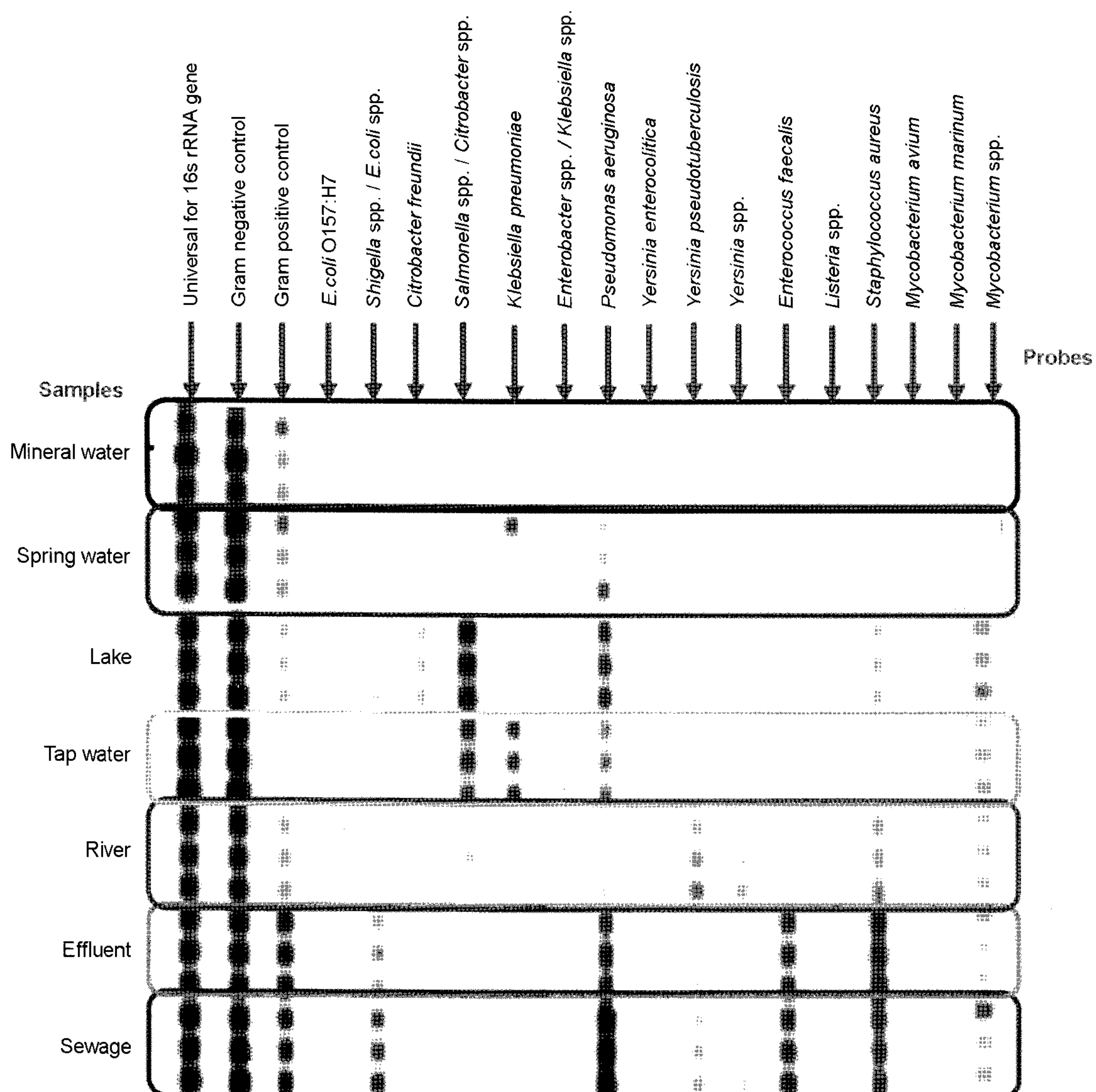

Fig. 3. Application of the PCR-REBA for detection and identification of waterborne pathogens from diverse water samples. PCR and the hybridization condition are described in the text.

Table 2. Comparison of microorganisms detected using conventional culture-API tests and PCR-REBA

	Lake	River	Effluent	Sewage	Tap water	Spring water	Mineral water
Conventional method	*E. coli* *Shigella* spp. *C. freundii* *Pseudomonas* spp. *Y. pseudotuberculosis* *S. aureus*	*Pseudomonas* spp. *Y. pseudotuberculosis* *E. faecalis* *S. aureus*	*E. coli* *Shigella* spp. *Pseudomonas* spp. *Y. pseudotuberculosis* *S. aureus*	*E. coli* *Shigella* spp. *Pseudomonas* spp. *Y. pseudotuberculosis* *E. faecalis* *S. aureus*	*Shigella* spp. *Pseudomonas* spp. *K. pneumoniae*	–	–
PCR-REBA	*E. coli / Shigella* spp. *C. freundii* *Salmonella/Citrobacter* *P. aeruginosa* *Y. pseudotuberculosis* *S. aureus* *Mycobacterium* spp.	*P. aeruginosa* *Y. pseudotuberculosis* *Yersinia* spp. *E. faecalis* *S. aureus* *Salmonella/Citrobacter* *Mycobacterium* spp.	*E. coli / Shigella* spp. *P. aeruginosa* *Y. pseudotuberculosis* *S. aureus* *E. faecalis* *Mycobacterium* spp.	*E. coli / Shigella* spp. *P. aeruginosa* *Y. pseudotuberculosis* *Yersinia* spp. *E. faecalis* *S. aureus* *Mycobacterium* spp.	*E. coli / Shigella* spp. *P. aeruginosa* *K. pneumoniae* *Salmonella/ Citrobacter* *Mycobacterium* spp.	*K. pneumoniae* *P. aeruginosa*	–

(Fig. 2A and 2B). For this, the sensitivity of PCR was estimated by DNA obtained from serially diluted *E. coli* O157:H7 cultured cells. The results showed that the sensitivities of both PCRs were between 1.2×10^5 cfu/ml and 1.2×10^4 cfu/ml.

Subsequently, the sensitivity of the PCR-REBA using each PCR amplicons were compared (Fig. 2C and 2D). For that purpose, PCR amplicons from amplification of target DNA using 10 fold diluted *E. coli* O157: H7 chromosomal DNAs (Fig. 2A and 2B) were hybridized to the REBA membrane containing probe for *E. coli* O157:H7.

Unlike PCR results which showed similar sensitivity of the single PCR to the one-tube nested PCR, the sensitivity of the hybridization ranged between 1.2×10^4 cfu/ml and 1.2×10^3 cfu/ml (Fig. 2C and 2D) giving at least 10 times higher sensitivity of the PCR-REBA than PCR only. Thus, it seems that PCR method followed by hybridization of the products to the REBA would improve the detection sensitivity.

Application of PCR-REBA for detection of waterborne pathogens from diverse water samples

A total of seven water samples from diverse sources including tap water, spring water, mineral water, lake, river, effluent and sewage from Gangwon Province in Korea were collected, and handled according to guideline suggested by the Korea Ministry of Environment (Korea Ministry of Environment. 2002).

Subsequently, the water samples were used both for isolation of waterborne pathogens using conventional culturing method and for identification of waterborne pathogens using PCR-REBA (Fig. 3).

Waterborne pathogens isolated and identified from this study is summarized in the Table 2. As shown in the Table 2, diverse waterborne pathogens including *C. freundii*, *E. coli*, *E. faecalis*, *K. pneumoniae*, *P. aeruginosa*, *Shigella* spp., *S. aureus*, *Y. pseudotuberculosis* were isolated using conventional culture method. Meanwhile, most of bacteria isolated and identified by conventional methods were also identified by PCR-REBA. Moreover, some of bacteria such as *Mycobacteria* which are usually difficult to cultivate by using routine media which are often used for screening typical waterborne pathogens were also identified. The presence of mycobacteria which were not be able to be isolated by conventional method in this experiment were confirmed by isolating mycobacteria using specific media for mycobacteria. Therefore, it seems that the presence of waterborne pathogens in water samples can be monitored by using PCR-REBA.

DISCUSSION

At present, the conventional method for screening waterborne pathogens from environment samples requires 3~7 days and also has a low sensitivity. In this, study, we developed PCR-REBA for detection of waterborne pathogens, and subsequently the sensitivity and the specificity of the PCR-REBA for detecting waterborne pathogens from

environment sources were compared to those of culture.

The PCR-REBA certainly satisfies requirements for simultaneous detection system of microbial contaminants in water than other molecular methods. It is more convenient, less expensive, and easier to perform because it uses commonly available reagents, less expensive equipment, and can analyze 45 samples within a single run. The PCR-REBA format uses a non precipitating ECL substrate; hence, it has the advantage in that nylon filters can be stripped and successfully reused at least 5 times and has significant impact in reducing the cost to minimal (Kohara et al., 2002).

Although relatively simple in concept, PCR-REBA is a powerful tool for detection and characterization of pathogen (Xiang et al., 2002; Zwart et al., 2003). Direct detection of nucleic acids from bacteria is feasible, but may lack the level of sensitivity needed for routine screening of environmental samples. When the amount of nucleic acid is not limiting, however, microarrays may prove very valuable as a fingerprinting tool and as a tool for marker discovery. When coupled to PCR, PCR-REBA have detection sensitivity equal to conventional methods with the added flexibility needed for discriminating multiple PCR reactions and for pathogen detection based on 16S rRNA sequences.

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